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# Neutrophil spontaneous death is mediated by down-regulation of autocrine signaling through GPCR, PI3K $\gamma$ , ROS, and actin

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Neutrophil spontaneous apoptosis plays a crucial role in neutrophil homeostasis and the resolution of inflammation. We previously established Akt deactivation as a key mediator of this tightly regulated cellular death program. Nevertheless, the molecular mechanisms governing the diminished Akt activation were not characterized. Here, we report that Akt deactivation during the course of neutrophil spontaneous death was a result of reduced PtdIns(3,4,5)P3 level. The phosphatidylinositol lipid kinase activity of PI3K $\gamma$ , but not class IA PI3Ks, was significantly reduced during neutrophil death. The production of PtdIns(3,4,5)P3 in apoptotic neutrophils was mainly maintained by autocrinely released chemokines that elicited PI3K $\gamma$  activation via G protein-coupled receptors. Unlike in other cell types, serum-derived growth factors did not provide any survival advantage in neutrophils. PI3K $\gamma$ , but not class IA PI3Ks, was negatively regulated by gradually accumulated ROS in apoptotic neutrophils, which suppressed PI3K $\gamma$  activity by inhibiting an actin-mediated positive feedback loop. Taken together, these results provide insight into the mechanism of neutrophil spontaneous death and reveal a cellular pathway that regulates PtdIns(3,4,5)P3/Akt in neutrophils.

Akt | apoptosis | reactive oxygen species

Neutrophils are the most abundant cell type among circulating white cells and are the major players in the innate immune system. Neutrophils are terminally differentiated and normally have a very short lifespan (7–20 hr) in circulation and in tissue (1–4 days) (1). The daily turnover of human neutrophils is  $0.8\text{--}1.6 \times 10^9$  cells per kg of body weight. The same number of neutrophils need to die to keep cellular homeostasis under physiologic condition.

Neutrophils die even in the absence of any extracellular stimuli; thus, this type of death is also called spontaneous death. It shares many features of classical apoptosis, such as cell body shrinkage, cellular crenation, exteriorization of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, vacuolated cytoplasm, mitochondria depolarization, nuclear condensation, and internucleosomal DNA fragmentation (2, 3). Neutrophil death can be modulated by various extracellular stimuli such as proinflammatory cytokines, cell adhesion, phagocytosis, red blood cells, and platelets. Under most conditions, neutrophils will be exposed to both pro- and antiapoptotic factors. The net effect on neutrophil death and survival reflects a balance between the activities of such factors. Constitutive neutrophil death is associated with up-regulation of death signaling and down-regulation of survival signaling. We recently reported that the activity of protein kinase B (PKB)/Akt, a well known prosurvival and antiapoptotic factor, decreases dramatically during the course of neutrophil death. Both PI3 kinase and Akt inhibitors enhance neutrophil death. Conditions delaying neutrophil death, such as treatment with GM-CSF, G-CSF, or IFN- $\gamma$ , restore Akt activity. Neutrophils depleted of PTEN, a phosphatidylinositol 3'-phosphatase that negatively

regulates Akt activity, live much longer than wild-type neutrophils (4, 5). However, the molecular mechanisms by which PtdIns(3,4,5)P3/Akt activity is down-regulated during neutrophil spontaneous death remain ill defined.

In the present study, we identified an autocrine signal pathway that is involved in the down-regulation of PtdIns(3,4,5)P3/Akt activity during neutrophil spontaneous death. Our data demonstrate that the activity of Akt in apoptotic neutrophils is mainly maintained by autocrinely released chemokines that elicit PI3K $\gamma$  activation via G protein-coupled receptors. Reactive oxygen species accumulated in apoptotic neutrophils, by blocking an actin-mediated positive feedback loop, serve as a physiological negative regulator of PI3K $\gamma$  and the subsequent PtdIns(3,4,5)P3 production and Akt activation.

## Results

**Akt Deactivation During the Course of Neutrophil Spontaneous Death Is a Result of Reduced PtdIns(3,4,5)P3 Level.** We have demonstrated that Akt deactivation is a causal mediator of neutrophil spontaneous death, but the molecular mechanisms by which Akt activity is down-regulated have not been fully investigated (4). Akt activation relies on its membrane translocation mediated by its specific association with PtdIns(3,4,5)P3 on the plasma membrane. Only the Akt molecules on the plasma membrane can be phosphorylated and activated. The level of active Akt (phospho-Akt) drastically declines during neutrophil death, whereas total Akt does not change (Fig. 1A–C), suggesting that the decrease of Akt activity is not a result of protein degradation. Akt membrane translocation and subsequent activation was previously thought to depend solely on concentrations of PtdIns(3,4,5)P3 in the membrane (6, 7). Recently, we demonstrated that two inositol phosphates, InsP7 and Ins(1,3,4,5)P4, compete for Akt-PH domain binding with PtdIns(3,4,5)P3 both in vitro and in vivo, providing another level of regulation for Akt membrane translocation and activation (8, 9). However, the levels of InsP7 and Ins(1,3,4,5)P4 are extremely low in unstimulated neutrophils, suggesting that the decreased Akt activation is likely caused by the decrease of PtdIns(3,4,5)P3 production (8) (Fig. S1). To confirm this, we measured the level of PtdIns(3,4,5)P3. Our results show that during the course of neutrophil death, levels of PtdIns(3,4,5)P3 decrease dramatically, whereas levels of PtdIns(4,5)P2, the substrate of PtdIns

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The authors declare no conflict of interest.

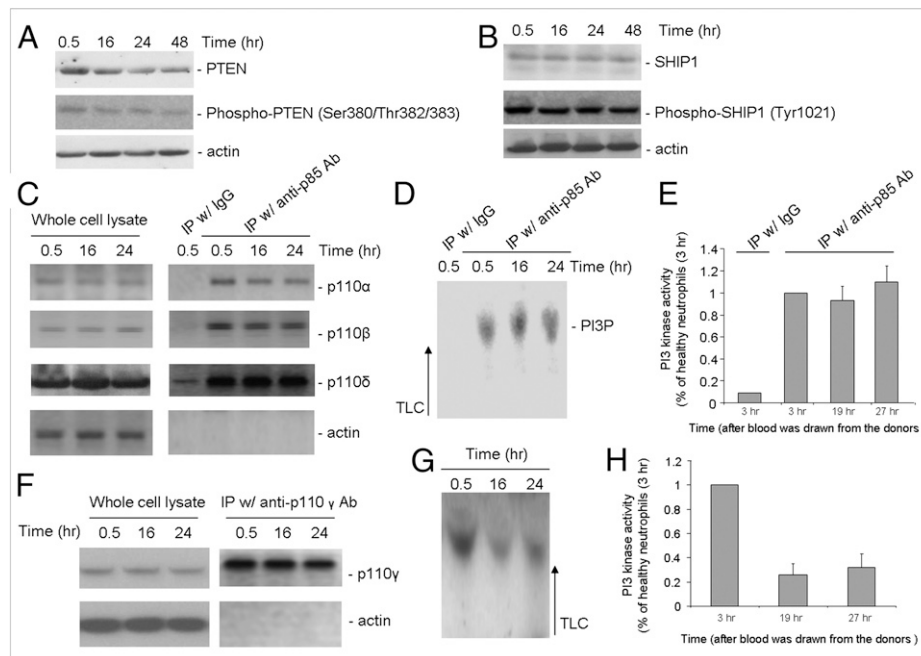
\*This Direct Submission article had a prearranged editor.

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**Fig. 2.** Deactivation of PtdIns(3,4,5)P3/Akt signaling during neutrophil spontaneous death is a result of reduced PI3K $\gamma$  activity. (A) The level of PTEN in normal and apoptotic neutrophils. Shown is the result of a representative experiment that was repeated three times. (B) The level of SHIP in normal and apoptotic neutrophils. Total and phosphorylated SHIP were detected by Western blot using anti-SHIP and anti-phospho-SHIP antibodies, respectively (Cell Signaling). Shown is the result of a representative experiment that was repeated three times. (C–E) The enzymatic activity of PI3K class IA is not altered during neutrophil spontaneous death. (C) The three isoforms of PI3K class IA were pulled down with a PI3K p85 antibody (Upstate Biotechnology). Neutrophil whole-cell lysates and immunoprecipitated samples were blotted with indicated PI3K antibodies. Shown is the result of a representative experiment that was repeated three times. (D) PI3 kinase activity of the immunoprecipitated enzymes. Shown is the result of a representative TLC plate. At each indicated time point, the kinase reaction was stopped and the lipids were extracted and analyzed by TLC. The positions of individual phosphatidylinositol were assigned from their migration distance matching those of corresponding authentic  $^{32}$ P-labeled standards. The amount of  $^{32}$ P-labeled PtdIns(3)P was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics). The PI3 kinase activity was expressed as the percentage of activity at time 3 hr (healthy neutrophils). (E) The data of the densitometric analyses are expressed as the percentage of the normal neutrophil control (3 hr). All values represent mean  $\pm$  SD of three separate experiments. (F and G) The enzymatic activity of PI3K class IB (PI3K $\gamma$ ) is down-regulated during neutrophil spontaneous death. (F) PI3K $\gamma$  was pulled down with a p110 $\gamma$  antibody (Upstate Biotechnology). Neutrophil whole cell lysates and immunoprecipitated samples were blotted with a rabbit polyclonal anti-p110 antibody. Shown is the result of a representative experiment that was repeated three times. (G) PI3 kinase activity of the immunoprecipitated enzymes. Shown is the result of a representative TLC plate. (H) The data of the densitometric analyses are expressed as the percentage of the normal neutrophil control. All values represent mean  $\pm$  SD of three separate experiments.

control cells cultured in the presence of serum (Fig. 3A). This result is consistent with the fact that class IA PI3Ks are not involved in regulating PtdIns(3,4,5)P3 signal during neutrophil death (Fig. 2).

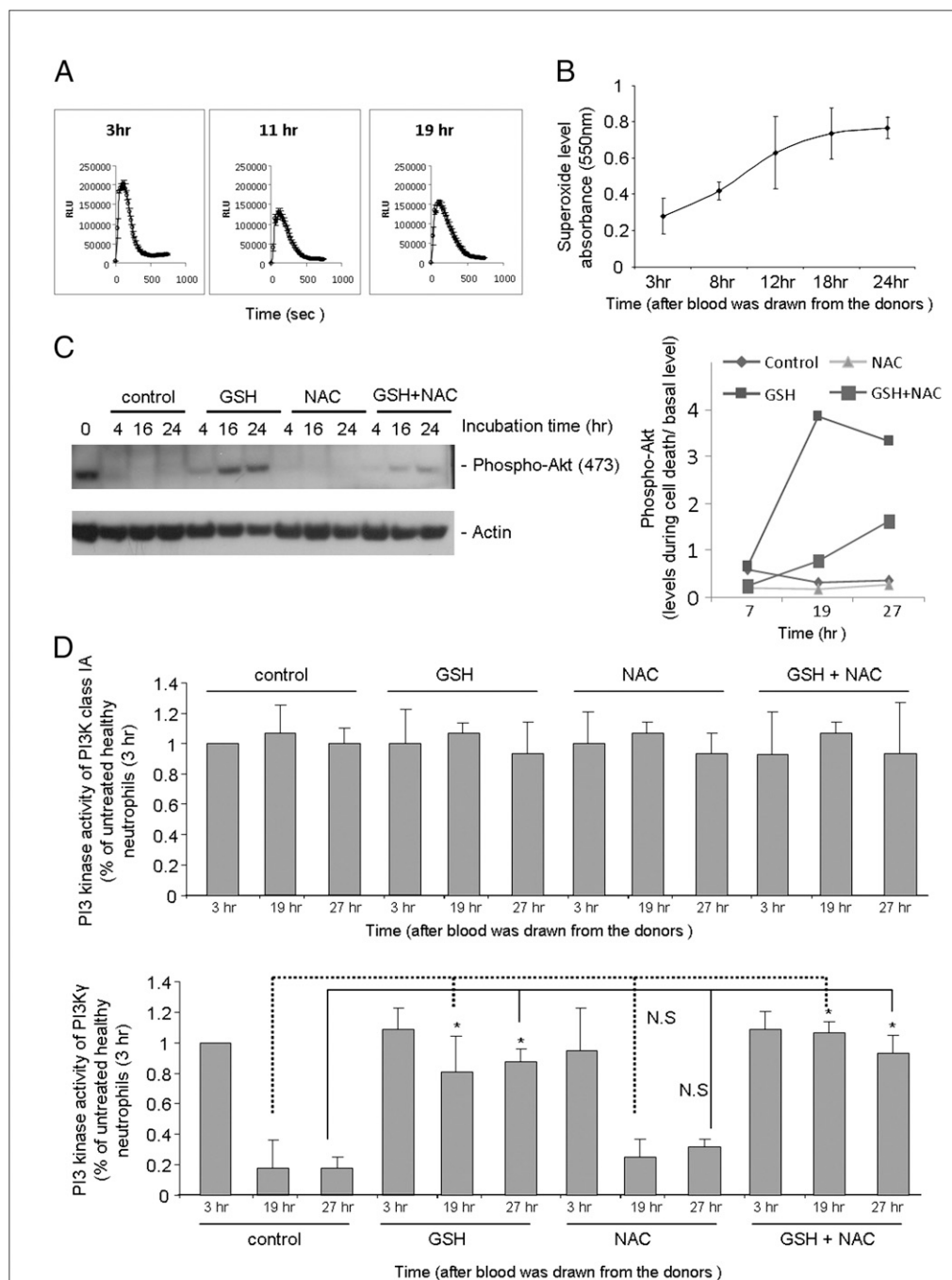
In neutrophils, PtdIns(3,4,5)P3 signal can also be elicited by heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins)-coupled receptors. Chemokines bind receptors on cell membrane and induce the dissociation of a specific G protein into  $\alpha$  and  $\beta\gamma$  subunits. Released  $\beta\gamma$  subunits are able to directly initiate activation class IB PI3K (PI3K $\gamma$ ) (1, 6). We have shown that deactivation of PI3K $\gamma$ , but not class IA PI3Ks, was responsible for Akt deactivation during neutrophil death, suggesting the involvement of GPCR-mediated pathways in regulating neutrophil death. We explored the role of GPCR using a bacterial-derived toxin, pertussis toxin (PTX), which catalyzes ADP ribosylation of G proteins and thus suppresses their activation. We found a significant accelerated death in PTX-treated neutrophils (Fig. 3B). At 9 hr in culture, PTX-treated neutrophils showed nearly one fold higher death rate than untreated neutrophils. Similar results were obtained at 15 and 24 hr. The difference became nonsignificant at 48 hr, because most untreated neutrophils also became apoptotic.

Because serum deprivation did not affect the half-life of neutrophils, the factors leading to GPCR activation might be produced by the cultured neutrophils in an autocrine manner. To test this, we cultured neutrophils in serum-free medium and

examined the secreted “GPCR activating activity” in the supernatants (Fig. 3C). Uniform treatment of freshly isolated neutrophils with chemokines or formyl-peptide (e.g., fMLP) elicits instant GPCR activation and elevation of PtdIns(3,4,5)P3 in the plasma membrane (17). We evaluated GPCR-elicited PtdIns(3,4,5)P3 signaling by measuring the level of endogenous Akt phosphorylation. Before chemoattractant stimulation, Akt phosphorylation was virtually undetectable in neutrophils (5). Upon stimulation, neutrophils showed maximum Akt phosphorylation at 2 min, which then declined marginally by 5 min. We used the level of Akt phosphorylation at 3 min after stimulation to assess GPCR activation. Our results showed that a large amount of “GPCR activating activity” was secreted and accumulated in the culture medium (Fig. 3C). Its ability to induce Akt phosphorylation was completely inhibited by PTX, further demonstrating that the activity of these secreted factors was indeed mediated by GPCR. Supporting this autocrine chemokine release mechanism, a significant amount of CXC chemokine IL8 was detected in the neutrophil culturing medium (Fig. 3D). IL8 can bind and activate G protein-coupled CXCR1 and CXCR2 receptors leading to activation of PI3K $\gamma$  and Akt (18). Thus, IL8 should be one of the neutrophil-released chemokines that support neutrophil survival. However, numerous chemokines can be produced by neutrophils; it is unlikely that IL8 will be the only one playing a role in neutrophil spontaneous death.







**Fig. 4.** ROS production is required for deactivation of PI3K $\gamma$  in neutrophil spontaneous death. (A) Aging neutrophils can still produce ROS. Human neutrophils were cultured for indicated periods of time and stimulated with 100 nM fMLP ( $10^5$  cells per 200  $\mu$ L per well). ROS production was monitored in the presence of 50  $\mu$ M isoluminol and 0.8 U of HRP in a luminometer at 37  $^{\circ}$ C. Chemiluminescence (arbitrary light units) was recorded (for 2 sec) at indicated time points after the addition of fMLP. Data are mean  $\pm$  SD from one experiment representative of three. (B) Reactive oxygen species accumulate during the course of neutrophil spontaneous death. Human neutrophils ( $10^7$  per data point) were cultured for indicated periods of time. The cells were then filter-lysed through two layers of 5- $\mu$ m filter membrane, and the cytosolic ROS levels were assessed using cytochrome c. The absorbance (550 nm) represents the level of superoxide ion in each sample. All values were normalized to the number of intact cells ( $PI^-$  cells). Shown are means  $\pm$  SD of three independent experiments. (C) Antioxidant reagents enhance the level of phosphorylation of endogenous Akt during neutrophil spontaneous death. Neutrophils were cultured in the presence of GSH (5 mg/mL) and/or NAC (0.2 mM) for indicated periods of time. Total and phosphorylated Akt were detected by Western blot as described (5). All samples were normalized to the amount of total Akt. basal level, level of phospho-Akt at time 3 hr. (D) Antioxidant reagents enhance the PI3 kinase activity of PI3K $\gamma$  but not PI3K class IA enzymes during the course of neutrophil spontaneous death. Neutrophils were cultured in the presence of indicated antioxidants as described above. The PI3 kinase activities of immunoprecipitated PI3K class IA and PI3K $\gamma$  were analyzed as described in Fig. 2. All values represent mean  $\pm$  SD of three separate experiments. \*,  $P < 0.001$  versus untreated cells at the same point by Student's  $t$  test. N.S., not significant.

period. It is well known that disruption of cortical F-actin in neutrophils will augment degranulation and release of chemo-

kines, which might be responsible for the early elevation of PtdIns(3,4,5)P3 signal in the treated cells.





# Supporting Information

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## SI Discussion

Constitutive neutrophil death is an important mechanism for modulating neutrophil homeostasis. Accelerated neutrophil death leads to a decrease of neutrophil counts (neutropenia), augments the chance of contracting bacterial or fungal infections, and impairs the resolution of such infections. However, delayed neutrophil death elevates neutrophil counts (neutrophilia), which is often associated with bacterial infection, myeloid leukemia, and acute myocardial infarction. Neutrophil death is also an essential cellular event for maintaining neutrophil number in infection and inflammation. Neutrophils are recruited to the infected tissues to engulf, kill, and digest invading microorganisms. However, the enzymes and reactive oxygen species (ROS) released by neutrophils can also damage the surrounding tissues. To prevent senescent neutrophils from releasing their toxic contents, these cells become apoptotic and are then recognized, engulfed, and cleared by professional phagocytes such as tissue macrophages. This safe clearance provides a mechanism of reducing the number of viable and activated neutrophils without releasing the potentially harmful enzymes and ROS, thereby facilitating the resolution of inflammatory response. Delayed death and clearance of neutrophils in tissues causes unwanted and exaggerated inflammation. Thus, the death program in neutrophils needs to be well controlled to provide a nice balance between their immune functions and their safe clearance (1–4).

In this study, we identified ROS as a key regulator of PI3K $\gamma$  in neutrophils. ROS have been implicated in a variety of cell death processes and are also recognized as one of the causal mediators of neutrophil spontaneous death. ROS accumulate during the course of neutrophil spontaneous death, and their pro-death activity is likely mediated by multiple pathways and mechanisms. ROS may lead to DNA alteration and trigger p53, which classically induces apoptosis following genotoxic injury (5, 6). Alternatively, ROS may directly alter the activity of intracellular signaling pathways involved in neutrophil death/survival such as NF- $\kappa$ B and MAPK (7–9). In neutrophils, it was also shown that death receptor clustering and the subsequent activation of caspase-8 are the results of ROS-dependent ceramide generation and may occur independently of Fas ligation in spontaneous death (10, 11). The cytotoxic free radical level can also be elevated by nitric oxide synthase (NOS)-mediated NO production. Exogenous nitric oxide and physiologically relevant NO donors, such as S-nitrosoglutathione, SIN-1, SNP, and GEA3162 significantly enhanced neutrophil apoptosis (12–16). Interestingly, high levels of ROS or reactive nitrogen species (RNS) inhibit caspase activity, indicating that an alternative caspase-independent death pathway may be involved in ROS-induced cell death (17, 18). It was reported that oxidative stress can trigger endonuclease G-mediated DNA fragmentation in the absence of caspase activity, providing a possible caspase-independent death pathway mediating ROS-induced neutrophil death (19).

The current study provides a mechanism by which ROS induce apoptosis in neutrophils, namely by inhibiting actin polymerization and subsequent amplification of the prosurvival PI3K/Akt pathway. How do ROS inhibit actin polymerization? In recent years, ROS has been identified as an important second messenger that can regulate intracellular signal transduction under a variety of physiological and pathophysiological conditions. During respiratory burst or oxidative stress, it is becoming increasingly clear that intracellular signal transduction gets altered (20–22). Such redox regulation of cell signaling involves modification of reactive thiols on specific cysteine residues of proteins, converting

them from a reduced to an oxidized form (23–26). In recent years, an increasing number of thiol-containing proteins have been identified to use ROS as a mediator to regulate their function. Most importantly, many of these thiol modifications are reversible, ensuring that normal protein function can be restored upon release of oxidative stress or termination of oxidative burst. The major types of thiol modifications that have been shown to play an important redox dependent role include glutathionylation, sulfenic acid formation, nitrosylation, and disulfide bond formation. Many cellular targets such as protein tyrosine phosphatases, protein tyrosine kinases, integrins, and Ras, have been identified, and ROS could regulate actin polymerization indirectly by modulating these targeted signal molecules. Alternatively, ROS may also directly modify actin. Monomeric G-actin is a cytosolic protein that continuously polymerizes and depolymerizes from a filamentous F-actin polymer in an ATP-powered cycle (27). Numerous studies have shed light on the functionality and mechanisms underlying actin glutathionylation. In-vitro actin polymerization assays demonstrated that glutathionylated actin polymerizes inefficiently in comparison with unglutathionylated actin (24, 28, 29).

We identified a ROS-mediated intracellular mechanism that regulates actin polymerization and subsequent amplification of PtdIns(3,4,5)P3 signaling. Because actin is involved in a variety of cellular functions such as migration, polarization, and cell adhesion, it will be intriguing to see whether ROS also play a role in these cellular processes. In addition, it will be important to examine whether other downstream targets of PtdIns(3,4,5)P3, such as GSK3, BAD, PDK1, and Foxo, as well as the related cellular functions can also be regulated by ROS.

## SI Materials and Methods

**Human Primary Neutrophils.** We isolated human primary neutrophils from discarded white blood cell filters (WBF2 filter; Pall Corporation), which were provided by the Blood Bank Lab at the Children's Hospital, Boston. Neutrophils were purified using a standard protocol. Briefly, erythrocytes were sedimented by adding an equal volume of dextran/saline solution (3% dextran T-500 in 0.9% NaCl) at room temperature for 25 min. The erythrocyte-depleted supernatants were then layered on Lymphocyte Separation Medium (1.077 g/mL Ficoll–Hypaque solution; Voigt Global Distribution) and centrifuged at 400  $\times$  g at room temperature for 20 min. Contaminated erythrocytes in the neutrophil pellets were lysed after a brief (<30 sec) treatment with 0.2% NaCl. Neutrophils were then resuspended in RPMI medium 1640 containing 10% heat-inactivated FBS at a density of  $4 \times 10^6$  cells per mL and maintained at 37  $^{\circ}$ C. The purity of neutrophils was >97% as determined by both Wright–Giemsa staining and FACS analysis with CD15 antibody. We routinely obtain about  $1\text{--}3 \times 10^8$  neutrophils from one filter (450 mL of blood from a healthy donor). We have compared the neutrophils that we collected through filter with those obtained by vein puncture and stored in anticoagulant testing tubs, and found that the filtration method does not impair neutrophil function (e.g., chemotaxis and the time course of cell death). All blood is drawn from healthy blood donors.

**PI3K Assays.** Human primary neutrophils were cultured in 35-mm dishes at a density of  $4 \times 10^7$  cells per mL per plate. At each time point, cells ( $\sim 3 \times 10^7$  cells per data point) were lysed in 200  $\mu$ L of lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 1.5 mM  $\text{Na}_3\text{VO}_4$ , 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM  $\beta$ -glycerophosphate,



5  $\mu\text{g/mL}$  aprotinin, 1  $\mu\text{g/mL}$  leupeptin, 6  $\mu\text{g/mL}$  chymostatin, 0.7  $\mu\text{g/mL}$  pepstatin, 1 mM DFP, 1 mM PMSF). The samples were centrifuged for 10 min to sediment insoluble material. The supernatants were transferred to new tubes, and incubated with 5  $\mu\text{L}$  of anti-PI3 kinase antibody (p85 antibody or p110 $\gamma$  antibody; Upstate Biotechnology) for 1 hr at 4 °C. Protein A-agarose beads (60  $\mu\text{L}$  of 50% slurry) were added to each tube and incubated with mixing for another hour at 4 °C. Immunoprecipitated enzymes were collected by centrifuging for 5 sec and washed three times with freshly prepared reaction buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.1 mM  $\text{Na}_3\text{VO}_4$ ). The kinase assay was carried out at 30 °C for 15 min in a 50- $\mu\text{L}$  reaction containing 10 mM  $\text{MgCl}_2$ , 10  $\mu\text{M}$  ATP, 20 mM Hepes (pH 7.5), 20  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP, and 0.1 mg/mL phosphatidylinositol. The reaction was stopped with 100  $\mu\text{L}$  of ice-cold 1 M HCl. The lipids were extracted with 2 mL of chloroform:methanol (1:1). After centrifugation at 1,000 rpm for 5 min, the lower organic phase was isolated and further extracted with 2 mL of methanol:1 M HCl (1:1). The lower phase was then isolated and dried under nitrogen gas. Silica gel 60 TLC plate (VWR) was prerun overnight with 1.2% potassium oxalate (Sigma) in  $d\text{H}_2\text{O}$ :methanol (3:2) and then dried and heat-activated in an oven (100 °C) for 3 min. The dried lipid samples were resuspended in 30  $\mu\text{L}$  of chloroform:methanol (2:1) and 10  $\mu\text{L}$  was spotted. TLC was performed using chloroform:acetone:methanol:acetic acid: $d\text{H}_2\text{O}$  (30:12:10:9:6) as a mobile phase. After the solvent front reached the top, the plate was taken out, dried, and analyzed by autoradiography.

**FACS Analysis of Neutrophil Spontaneous Death.** Neutrophils were cultured for the indicated time and stained using an Annexin V detection kit (Caltag Laboratories) following a protocol provided by the manufacturer. FACS was performed using a FACSCanto II flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Ten thousand cells were collected and analyzed using BD FACSDiva software (Becton Dickinson).

**Western Blot analysis.** Neutrophils were kept at  $4 \times 10^6$  cells per mL in the spontaneous death assay. At each indicated time point, neutrophils (4 million cells per data point) were spun down and lysed immediately with 100  $\mu\text{L}$  of boiling protein loading buffer (Invitrogen). Samples were incubated at 100 °C for 5 min and transferred on ice. After a brief sonication (5–10 sec), 25  $\mu\text{L}$  of lysate was used for Western blot analysis. For Western blotting, a 4–20% SDS/PAGE system (Invitrogen) was used for protein separation, and an ECL Western blotting kit (Amersham) was used for protein detection.

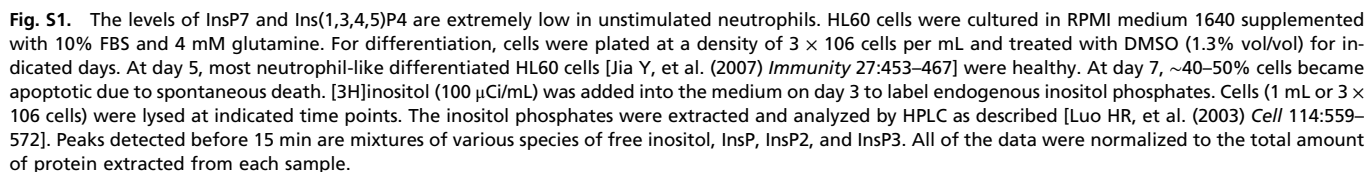
**Release of IL-8 Chemokine by in Vitro-Cultured Neutrophils.** Human primary neutrophils were cultured in 35 mm-dishes at a density of  $4 \times 10^7$  cells per mL per plate for 30 min and washed with RPMI medium 1640–1% BSA three times. Cells were resuspended in 500  $\mu\text{L}$  of RPMI 1640–1% BSA and then transferred to a 1.5-mL Eppendorf tube. Supernatants were collected at indicated times and secreted IL-8 chemokines was measured by an ELISA kit following a protocol provided by the manufacturer (R&D Systems).

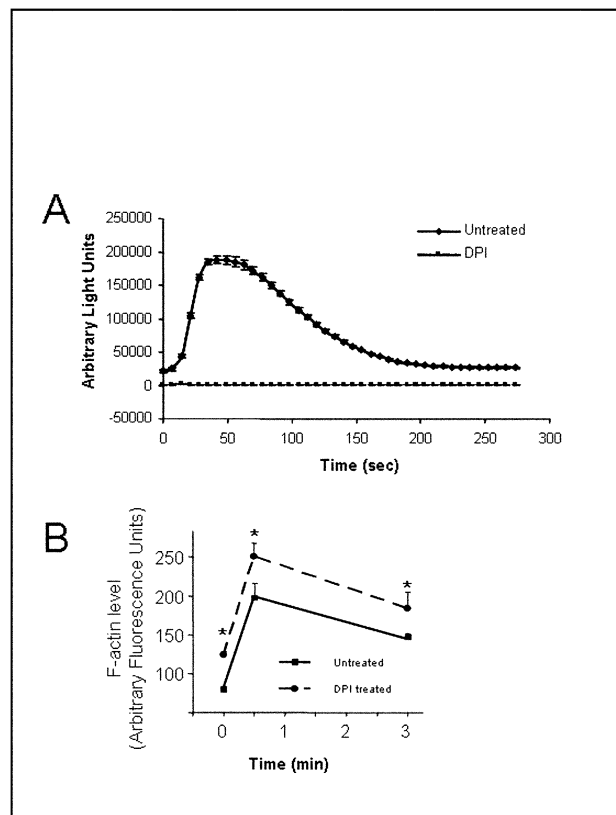
**NADPH Oxidase Activity Assay.** Superoxide anions produced by NADPH oxidase were detected using isoluminol chemiluminescence (30). Neutrophils were resuspended at a density of  $10^7/\text{mL}$  in HBSS (containing  $\text{Ca}^{2+}/\text{Mg}^{2+}$  salts) and kept on ice until use. A reaction mixture containing 20  $\mu\text{L}$  of 0.5 mM isoluminol (TCI America), 10  $\mu\text{L}$  of 80 U/mL horseradish peroxidase (Type XII; Sigma), 40  $\mu\text{L}$  of cells, and 110  $\mu\text{L}$  of HBSS (containing  $\text{Ca}^{2+}/\text{Mg}^{2+}$  salts) was added into each well of a 96-well Maxisorp plate (Nunc) and allowed to equilibrate to 37 °C for 4 min in a 1420 Wallac Victor<sup>2</sup> multilabel counter. A prestimulus luminescence reading was taken (for 2 sec). Twenty microliters of 10 $\times$  concentrated fmlp was then added to the reaction mixture via the injection port of the luminometer and luminescence was recorded (for 2 sec) at fixed time intervals.

**Measurement of Total ROS Level in Neutrophils.** The levels of reactive oxygen species in neutrophils were assessed using a cytochrome-C assay as described (31). Briefly, freshly prepared human neutrophils ( $10^7$  per data point) were cultured for indicated periods of time, washed, and resuspended in 1 mL of HBSS containing 1.5 mg/mL cytochrome *c*. The cells were then filter-lysed through two layers of 5- $\mu\text{m}$  filter membrane. After 5 min at RT, cytochrome *c* reduction in each sample was detected by spinning-down cell debris and reading absorbance (at 550 nm) of the supernatant in a spectrophotometer. The absorbance represents the level of superoxide ion in each sample.

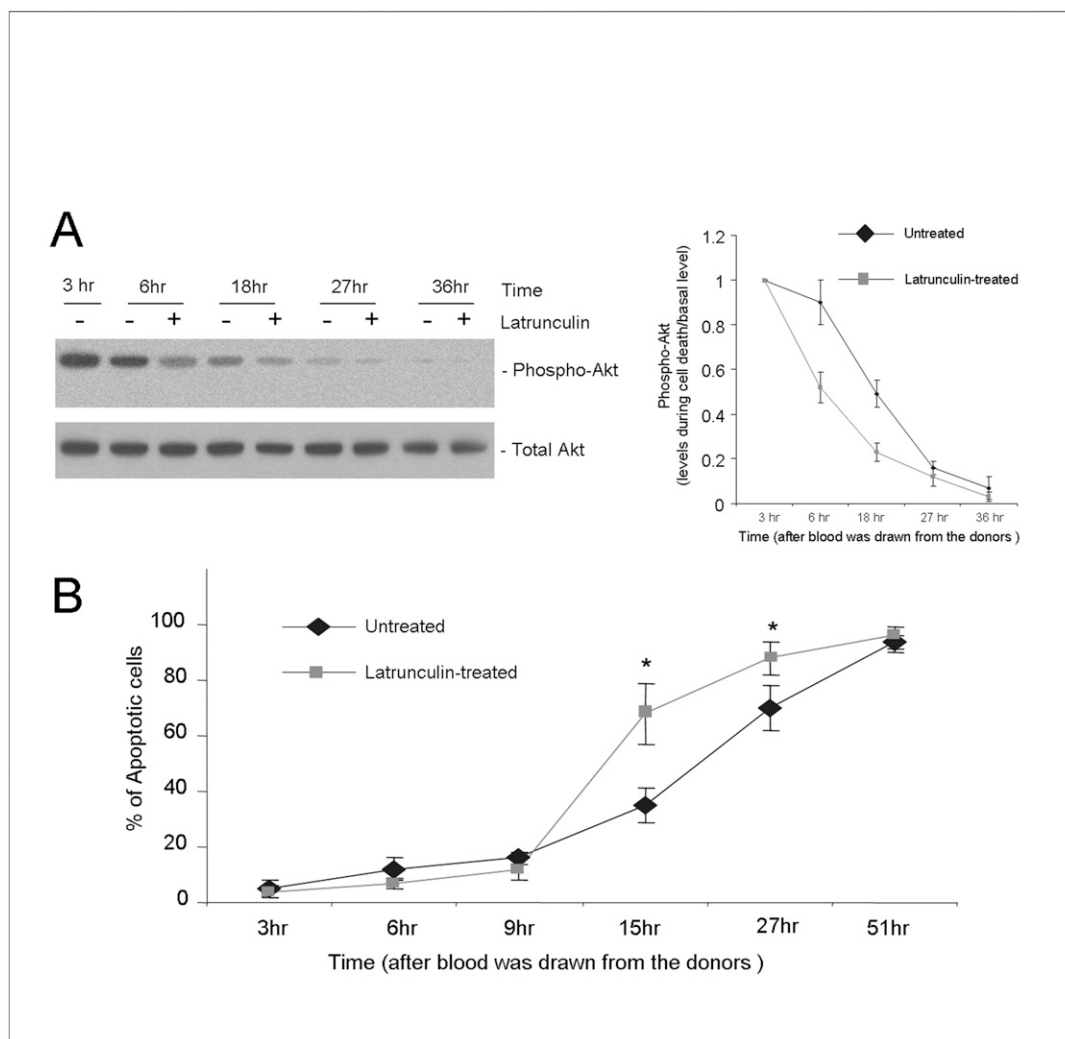
**Quantification of F-Actin Levels.** Human neutrophils were cultured at a density of  $5 \times 10^6/\text{mL}$  in RPMI/0.25% BSA. Cells ( $5 \times 10^5$ ) were stimulated with 100  $\mu\text{L}$  of 200 nM fMLP in RPMI/0.25% BSA for 1, 3, or 5 min, fixed with 200  $\mu\text{L}$  of 8% formaldehyde, and incubated on ice for 20 min. After preblocking overnight at 4 °C with 5% nonfat dry milk, cells were stained for 30 min with 0.13  $\mu\text{g/mL}$  fluorescein phalloidin (Sigma) in PBS containing 0.1% Triton X-100 and 5% milk. Intensity of phalloidin-staining was analyzed using a FACSCalibur machine.

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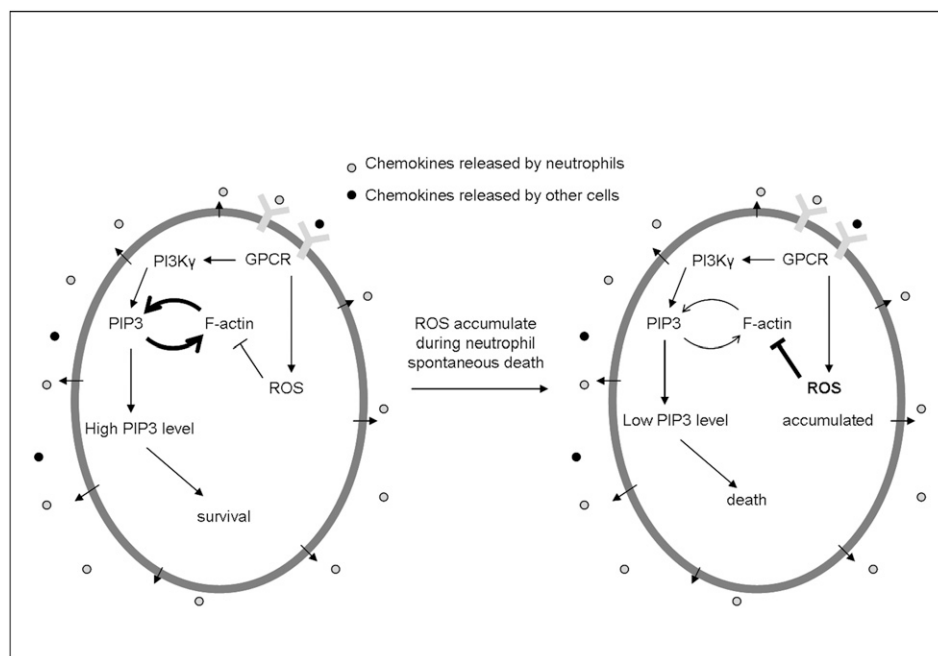


**Fig. S2.** ROS inhibit actin polymerization in neutrophils. (A) Chemoattractant-induced ROS production is suppressed by a specific pharmacological inhibitor of NADPH oxidase. Human blood neutrophils ( $5 \times 10^5$ ) were left untreated or treated with 50  $\mu\text{M}$  diphenyliodonium chloride (DPI) for 30 min at 37  $^{\circ}\text{C}$ . Cells were then stimulated with 100 nM fMLP and ROS production was monitored in the presence of 50  $\mu\text{M}$  luminol and 0.8 U of HRP in a luminometer at 37  $^{\circ}\text{C}$ . Chemiluminescence (arbitrary light units) was recorded (for 1 sec) at indicated time points. Data are mean  $\pm$  SD ( $n = 3$ ) from one experiment representative of three. (B) Actin polymerization in DPI-treated human neutrophils. Human neutrophils ( $0.5 \times 10^6$ ) treated with (or without) 50  $\mu\text{M}$  DPI and then stimulated with 10 nM fMLP. Cells were fixed at specified time points, permeabilized, and stained with rhodamine-phalloidin as described in ref. 5. Stained neutrophils were then analyzed by fluorescence-activated cell sorting (FACS). Data are represented as mean of median fluorescence  $\pm$  SD ( $n = 3$ ) from one experiment representative of three.



**Fig. S3.** ROS-induced Akt deactivation and neutrophil spontaneous death are mediated by actin. (A) Latrunculin A, an inhibitor of actin polymerization, reduces the level of phosphorylation of endogenous Akt during neutrophil spontaneous death. Neutrophils were cultured in the presence of 2  $\mu$ M Latrunculin A for indicated periods of time. Protein extracts were resolved on SDS/PAGE. Total and phosphorylated Akt were detected by Western blot using anti-Akt and anti-phospho-Akt (Ser-473) antibodies as described (31). Relative amounts of phosphorylated Akt were quantified using NIH Image software. All samples were normalized to the amount of total Akt. Basal signal, level of phospho-Akt at time 3 hr. Data presented are the means ( $\pm$  SD) of three independent experiments. (B) Latrunculin A enhances neutrophil death. Freshly prepared neutrophils (3 hr after blood was drawn from healthy donors) were treated with 2  $\mu$ M Latrunculin A (Calbiochem) for indicated periods of time. Cell viability was assessed by FACS analysis as described in Fig. 1. At least three separate experiments were carried out with a minimum of 100,000 cells counted per data point. Cell viability was determined as the ratio of live to total cell number. The results are the means of three independent experiments. Bars indicate mean  $\pm$  SD. \*,  $P < 0.001$  versus untreated cells by Student's  $t$  test.





**Fig. S4.** Neutrophil spontaneous death is mediated by down-regulation of autocrine signaling through G protein-coupled receptors, PI3K $\gamma$ , ROS, and actin.